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Effect of ethylene glycol on the phase transition kinetics of gluco- and galactocerebrosides

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The effect of different concentrations of ethylene glycol in water on the phase transition (metastable → stable state) of Gaucher's glucocerebroside, of bovine brain cerebroside type II (non hydroxy acyl chains only) and of *N*-palmitoylgalactocerebroside has been investigated. The phase transition and its kinetics were inferred from the thermograms at different heating and cooling rates and confirmed by FTIR spectra of the cerebrosides in the different states. The significance of the conformational differences of the glucose and of the galactose residues with respect to their solvation, and the subsequent effect on the intermolecular interactions and the phase transition is discussed.

Cerebrosides, containing predominantly saturated acyl chains [1] without 2-hydroxyl residues, are polymorphic having two solid-crystalline states and they show hysteresis upon heating and cooling [1–4].

It has been claimed that the complex phase behaviour depends mainly on the presence or absence of the 2-hydroxyl residues in the acyl chains but not on the sugar moiety [2,3,5]. However, the effectiveness of the 2-hydroxyl residue in elimination of existence of both metastable and stable states points towards the involvement of the other head group hydroxyl residues as well, in the structure determination of the different states.

The structural difference between the gluco and the galactocerebrosides is that in the former case all the OH residues on the pyranose ring are oriented in the direction of the ether bond with the spingosine base while in the case of galactocerebroside one OH is located on the other side of the pyranose ring. This arrangement is responsible for the differences in frequencies and amplitudes of the C-O stretching bands of the gluco- and galactocerebrosides both in the stable and in the metastable state and it affects also the amide II bands in the respective states [6]. The intermolecular interaction between the sugar containing head groups is expected to depend on their solvation. Curatolo [7] investigated the influence of the exchange of 50% of water by ethylene glycol or by dimethylsulfoxide on the thermotropic properties of the semisynthetic *N*-palmitoylgalactocerebroside.

In the present communication we report the effect of different concentrations of ethylene glycol on the exothermic transition of the metastable to stable state of four ceebrosides: Gaucher's glucocerebroside (GGlC) from two sources and the galactocerebrosides: *N*-palmitoylgalactocerebroside (NPGC) and bovine brain galactocerebroside type II fraction (BCII). The influence of solvation on the endothermic transition: rigid to liquid-

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crystalline state and of the exothermic transition upon cooling, is also discussed.

Experimental. *N*-Palmitoylgalactocerebroside and bovine brain galactocerebroside type II were purchased from Sigma, St. Louis, MO, U.S.A., Gaucher's glucocerebroside was a gift from Prof. Y. Barenholz, Hadassa Med. School, Jerusalem, Israel. Another sample of glucocerebroside was purchased form Sigma. The cerebrosides were weighted directly into the aluminum pans of the instrument and appropriate volumes of $5 \cdot 10^{-2}$ M KCl solution or is mixtures with ethylene glycol at pH 6–6.5 were added. The concentrationi of the lipids was between 4 and 10%. The DSC experiments were performed on Du Pont 990 Thermal Analyzer equipped with cell base II and homemade cooling equipment. Infrared spectra of cerebrosides dispersed in 33% ethylene glycol in water were measured using a Nicolet MX-1 FTIR spectrophotometer.

Results. In Figs. 1A–1C the thermograms of brain cerebroside type II (BCII), of Gaucher's glucocerebroside (GGlC) and of *N*-palmitoylgalactocerebroside (NPGC), respectively, are presented. The glycolipids were dispersed either in 50 mM aqueous KCl alone or with ethylene glycol added to the aqueous KCl. The percentage of ethylene glycol is indicated on the figure. It is evident from the figure that the exothermic peaks of the galactocerebrosides: NBGC and BCII are similar but they are distinctly different from those of glucocerebroside GGlC. As seen from the figure, in the case of the galactocerebrosides the exothermic peak is splitted at zero or low concentrations of ethylene glycol. The T_m of the lower temperature exothermic peak rises with increase in the concentration of ethylene glycol and eventually the splitted peak merges into one peak. In the case of NPGC above 40% ethylene glycol the T_m of the exothermic peak shifts up to the domain of the endothermic peak splitting it into two: one at lower T_m, corresponding to the melting of the metastable domain and the other with the usual T_m corresponding to the melting of the stable solid-crystalline domain. In the presence of 50% ethylene glycol similarly to the results of Curatolo [7], no exothermic peak is observed any more. The behaviour of brain galactocerebroside type II is in this respect similar eventhough the exothermic

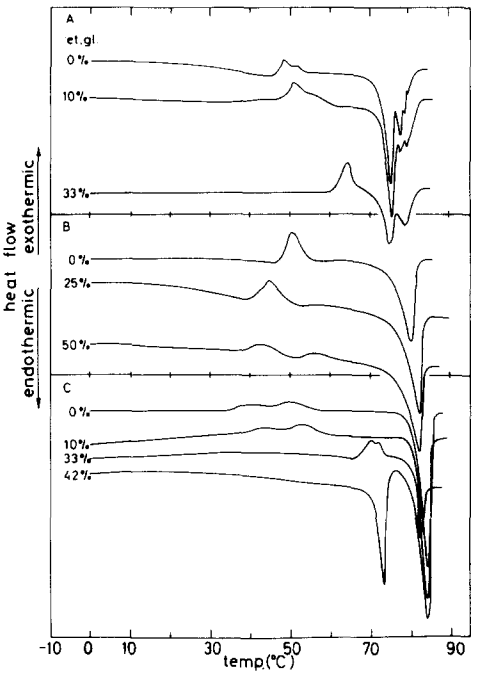

Fig. 1. Thermograms at 5 Cdeg/min of the cerebrosides: A, bovine brain type II; B, Gaucher's glucocerebroside; C, *N*-palmitoylgalactocerebroside. in the presence of 10- to 20-fold excess of different concentrations of ethylene glycol (et. gl.) in water as indicated on the thermograms.

peaks are less splitted and merge into one at 33% ethylene glycol. However the endothermic transitions are more complex, presumably because of the heterogeneous acyl chain composition and selective partial dehydration [8]. Ethylene glycol at high concentrations increases the transition enthalpy and induces hysteresis in phase transition of phospholipids [9]. The overall effect depends on the acyl chain composition but just like in the present case it is induced through the head group solvation and dehydration. Phospholipid head group dehydration resulting in rise in T_m is also induced by decreased water activity upon addition of different polyols [10]. Even at low concentrations of ethylene glycol, when the T_m of the exothermic peak is far below the endothermic T_m, multiple endothermic peaks are observed. At higher concentrations of ethylene glycol the elevated temperature peak with a T_m close to the transition of the anhydrous cerebroside becomes better separated and more pronounced (Fig. 1A

33% et.gl). Decreasing the scan rate at high concentration of ethylene glycol lowers as expected the T_m value of the exothermic peak. At the same time enhancement of the higher temperature endothermic peak of the presumably non hydrated form on account of the one with the lower T_m is observed (compare the thermograms at different scan rates in Fig. 2C). One can conclude from here that in the presence of ethylene glycol a non hydrated stable form coexists with the hydrated one and its fraction increases with the concentration of ethylene glycol. The dehydration of the stable state is a relatively slow process $t_{1/2} \approx 5-10$ min. The slow dehydration is in agreement with the observation that the hydration is interrelated with the lipid conformation and its variation [11]. The dehydration of the galactocerebrosides in the stable state in 33% ethylene glycol solution is also evident from the infrared spectra (Fig. 4). Short exposure to the temperature above the exothermic transition (approx. 73°C) yields amide I band spectra characteristic of the stable state [6]. Longer exposures yield spectra practically identical with the spectra of fresh unheated dispersions before complete hydration. Both glucocerebrosides yield under similar conditions spectra characteristic of the stable state [6].

The thermotropic behaviour of GGlC is also quite different. Only a single exothermic peak is observed in the absence of ethylene glycol. In contradistinction to the galactocerebrosides, T_m of this peak decreases with increasing concentration of ethylene glycol but above 25% of ethylene glycol a small second peak at higher temperature appears. The size of the second exothermic peak increases with further increase of ethylene glycol content. The infrared spectra of GGlC indicate that even at high concentrations of ethylene glycol it retains its hydration.

In Figs. 2A–2C thermograms of different cerebrosides in the presence of ethylene glycol at different scan rates are presented: (A), GGlC (prepared from the spleen of a Gaucher patient Hadassa Medical School, Jerusalem, by Professor Y. Barenholz) in the presence of 50% ethylene glycol (B), NPGC and (C) Type II in the presence of 33% ethylene glycol. The higher temperature exothermic peak of GGlC in 50% ethylene glycol is almost unaffected by the scan rate. However,

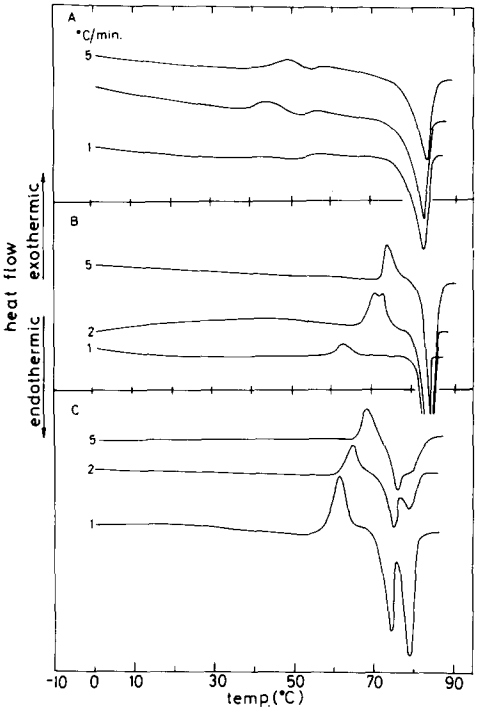

Fig. 2. Thermograms at different scan rates as indicated. A, Gaucher's glucocerebroside in 50% ethylene glycol in water; B, *N*-palmitoylgalactocerebroside in 33% ethylene glycol in water; C, bovine brain type II cerebroside in 33% ethylene glycol in water.

T_m of the low-temperature part of the peak and its size decrease with decreasing scan rate. It vanishes below 1 Cdeg/min (Fig. 2A, 1 Cdeg/min) and the major part of the metastable to stable phase transition seems to occur slow in a non cooperative mode at lower temperatures., In the case of GGlC from Sigma, eventhough the dependence of the exothermic peak location and shape on ethylene glycol concentration is similar, the dependence on the scan rate is somewhat different and the peak vanishes only at 0.5 Cdeg/min. The differences between the thermograms of the two GGlC's stem probably from the different acyl or sphingosine chain composition, which may vary from source to source.

The larger scan rate dependence of the T_m of the low temperature part of the split exothermic peak seems to be shared by all the cerebrosides. With increasing scan rate the T_m of the low-temperature peak components increases faster than

the T_m of the high-temperature component and the two merge eventually into one peak. This occurs at scan rates of 5 Cdeg/min for the brain type II galactocerebrosides at low concentration of ethylene glycol (at high concentration of ethylene glycol the peak is never splitted, Fig. 2C). The two components of the splitted peak of GGlC in 50% ethylene glycol merge at about 10 Cdeg/min.

In Fig. 3 the T_m of the exothermic peaks at scan rate of 5 Cdeg/min are plotted as a function of percentage of ethylene glycol in the dispersing solution. The increase of T_m of the exothermic peaks of the galactocerebrosides and the decrease of the T_m of glucocerebroside main exothermic peak with ethylene glycol concentration is seen. The T_m of the second peak of the glucocerebroside GGlC at around 60°C which appears at 25% ethylene glycol is also shown. The size of this peak increases with increase of the concentration of ethylene glycol, but its T_m does not change. For further understanding of the effect of solvation on the phase transition kinetics, cooling scan thermograms at different concentrations of ethylene gly-

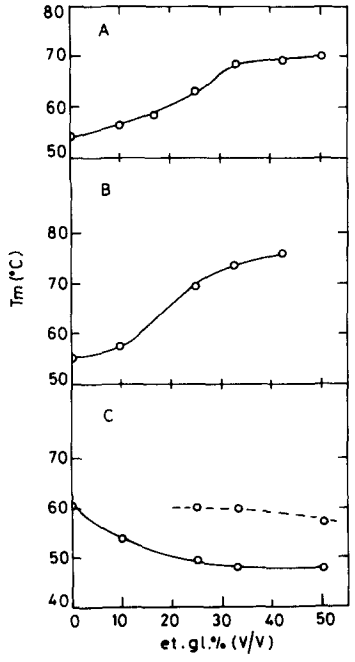

Fig. 3. T_m of the exothermic peak at 5 Cdeg/min as a function of the ethylene glycol in water content of the dispersing aqueous solution. A, *N*-palmitoylgalactocerebroside; B, bovine brain type II cerebroside; C, Gaucher's glucocerebroside.

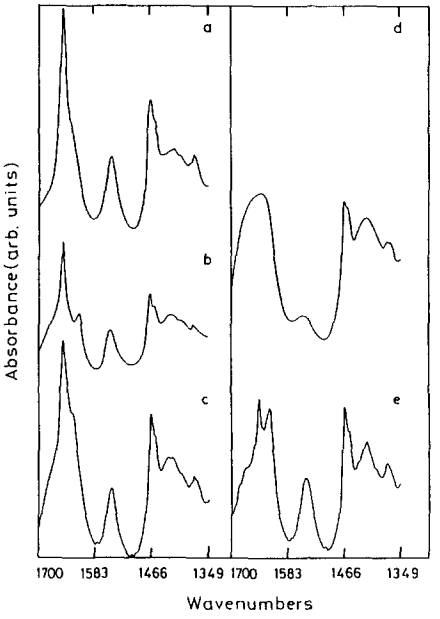

Fig. 4. Infrared spectra showing the amide 1 (approx. 1620–1650 wave number) and amide II (approx. 1540) bands for: a, BCII metastable; b, BCII stable obtained by exposure of the metastable form to 65°C for 30 s; c, BCII stable obtained by exposure of the metastable form to 65°C for 5 min; d, GGlC (from Haddassah Medical School) metastable; e, GGlC stable obtained by exposure of the metastable form to 70°C for 3 min. The metastables forms were obtained by heating to 85°C and quenching in cold water. BCII, brain cerebroside type II; GGlC, Gaucher's glucocerebroside.

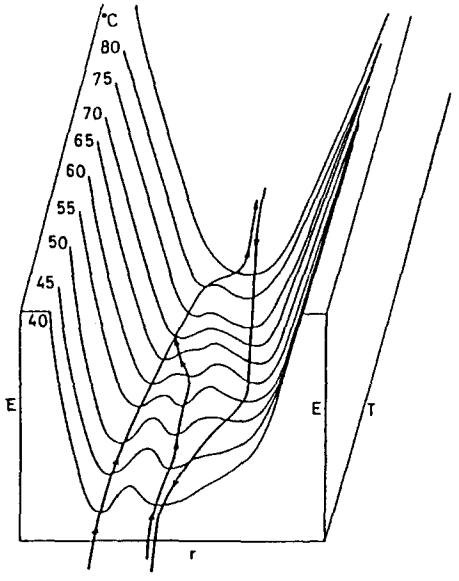

Fig. 5. Three-dimensional diagram of free energy profiles at different temperatures with potential barrier between one liquid-crystalline and two rigid phases.

col were carried out. In Fig. 6 there are the cooling scans of glucocerebroside (Sigma) and of *N*-palmitoylgalactocerebroside dispersed in water at scan rates between 5 Cdeg/min and 1 Cdeg/min. NPGC in water shows in agreement with the results of Curatolo [7] two peaks at the cooling scan. A narrow one with T_m changing very little (between 59°C and 59.5°C) but sharpening when changing the scan rate from 5 Cdeg/min to 1 Cdeg/min. A broad one, become narrower and changing its T_m for 51.5°C to 57.5°C for the same change in the scan rate. The glucocerebrosides in aqueous dispersions have only one transition peak from liquid to rigid-crystalline state which broadens slightly and its T_m is lowered by one degree when the scan rate increases from 1 Cdeg/min to 5 Cdeg/min. Bovine brain cerebroside type II and the GGlC extracted from the spleen of a Guacher's patient in Hadassah behave similarly with respect to the independence of the onset temperature on the scan rate. In every case, the transition from the liquid-crystalline phase to the rigid states is way below the melting temperature (undercooling) but starts above the temperature of relatively fast transition from the metastable to stable state. Thus the degree of transition to the stable state during cooling increases as the cooling rate decreases. Curatolo [7] suggested that the sharp peak at cooling of NPGC corresponds to the transition to the metastable and the broader one to the stable state. The independence of the onset temperature of the transition peak from the liquid-crystalline state on scan rate indicates that the nucleation occurs at a practically constant temperature of the undercooled sample, and the hysteresis loop is independent on scan rate. This corresponds to a steep dependence of the nucleation rate on temperature. Addition of ethylene glycol to water facilitates and narrows the hysteresis loops. The transition temperatures from the liquid-crystalline states at cooling increase as the scan rates decrease (Figs. 6 and 7). The temperature dependence is larger in the glucocerebroside than in the galactocerebrosides. The onset temperatures in 33% ethylene glycol vary from 70°C at 1 Cdeg/min till 62.5°C at 5 Cdeg/min for GGlC from

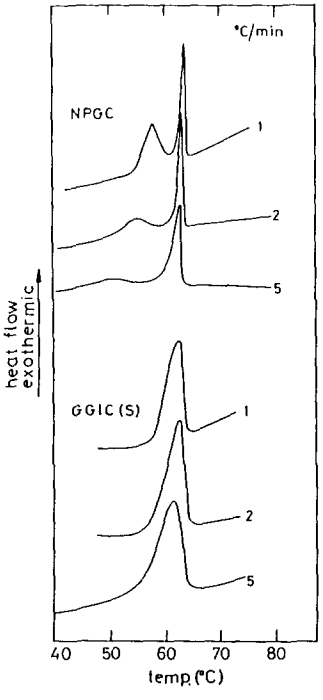

Fig. 6. Cooling scan thermograms of *N*-palmitoylgalactocerebroside (NPGC) and of Gaucher's glucocerebroside (GGlC) from Sigma dispersed in aqueous 0.05 M KCl at different scan rates as indicated.

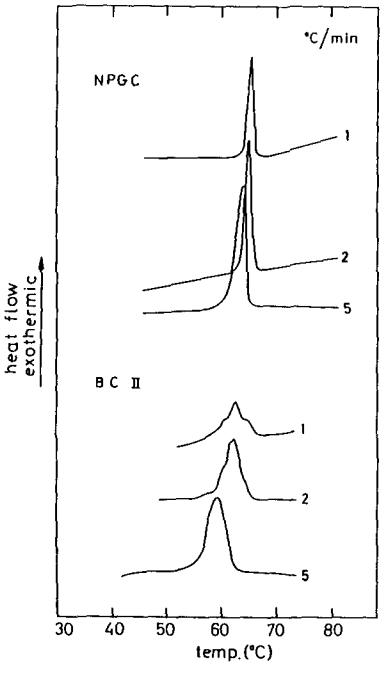

Fig. 7. Cooling scan thermograms of the galactocerebrosides *N*-palmitoylgalactocerebroside (NPGC) and brain cerebroside type II (BCII) dispersed in 33% ethylene glycol + 67% aqueous 0.05 M KCl at different scan rates as indicated.

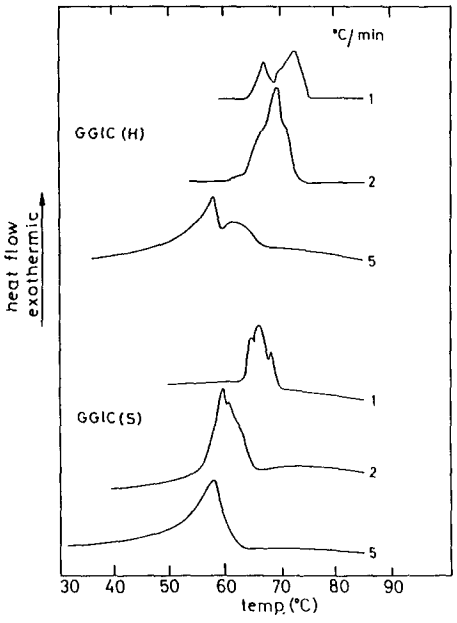

Fig. 8. Cooling scan thermograms of two samples of glucocerebrosides, one prepared in Hadassah Medical School from the spleen of a Gaucher's patient (GGlC-H) and one purchased from Sigma (GGlC-S) dispersed in 33% ethylene glycol and 67% 0.05 M aqueous KCl at different scan rates as indicated.

Sigma and from 75°C at 1 Cdeg/min till 66.5°C for GGlC from Hadassa Medical School. It is more difficult to compare the shift in T_m because of the splitting of the peaks, especially at low scan rates. Just like in the heating scan there is also a difference in the cooling scan thermograms of the different GGlC samples depending on the acyl chain composition and possibly also on the sphingosine base. There is a pronounced difference between the two gluco- and the two galactocerebroside samples. The onset temperatures of BBCII varies from 63°C to 66.5°C when the scan rate decreases from 5 Cdeg/min to 1 Cdeg/min. Those of NPGC vary from 64.5°C till 66°C.

Discussion. Ethylene glycol may affect the thermotropic properties of the cerebrosides through its effect on the activity of water and thus hydration and by direct participation in the solvation. The present evidences indicate that the dehydration effect of the rigid states is pronounced in the galactocerebrosides while the mixed solvation in the glucocerebroside. In either mechanism the effect is on the head groups including the sugar residues with a subsequent effect on the intermolecular interactions and on the transition between different states. The remarkable difference between the solvation tendencies of the glucose and the galactose residues and their subsequent interactions may shed light on the stereospecific interactions of the glycosphingolipids. The difference between the gluco and the galactocerebrosides seems to be in this respect kinetic. The transition of the less hydrated galactocerebrosides in the presence of ethylene glycol is impeded, while of the solvated glucocerebrosides is accelerated. The enthalpy of the exothermic transition (calculated at high scan rates) decreases in every case mildly with the ethylene glycol concentration in the dispersing solution, until in 33% ethylene glycol is about 2/3 of its value in water. At scan rates below 2 Cdeg/min the measured exothermic enthalpies start decreasing until at very low scan rates they vanish. Presumably there is a slow non cooperative transition below the peak temperatures, the heat capacity of whichis undiscernable from the base line. This is in agreement with the observations in the high sensitivity slow scan rate differential scanning calorimeters where at the very slow heating rates no metastable to stable transitions were observed [12]. Formation of metastable states can be eliminated if the cooling from the liquid-crystalline state is very slow. Indeed the exothermic peak at heating rate 5 Cdeg/min was partly or fully abolished after cooling at rates 2 Cdeg/min or lower. The transition is from the liquid-crystalline through the metastable to the stable rigid-crystalline state. The potential barrier between the stable and the metastable state giving rise to hysteresis can be depicted by three-dimensional energy profile diagrams [13]. It decreases with increasing temperature (Fig. 5) until the transition time is low enough to allow for the exothermic transition peaks (Figs. 1, 2). The transition between the liquid and the rigid-crystalline states shows hysteresis. The exothermic transition at cooling occurs at temperatures lower by about 20 Cdeg than the endothermic transition at heating and very close to the temperature of the exothermic transition from the metastable to the stable rigid state. If it occurs at temperature higher than the metastable-stable transition, then the

liquid-crystalline state converts directly into the stable rigid state. In the other case the conversion is through the metastable into the stable state and the completion of the conversion depends on the scan rate.

The melting of the rigid-crystalline states, either stable or metastable into the liquid-crystalline state occurs at the equilibrium melting temperature. The undercooling of the liquid-crystalline state, which requires nucleation before conversion into the rigid state, is responsible for the hysteresis loop.

We propose that the nucleation and its rate are related to dehydration upon transition from the liquid-crystalline to the metastable or to the stable rigid-crystalline phase. There are only about three unfreezable water molecules per molecule of the glucocerebroside [11] and about four water molecules per NPGC [14]. The difference between the stable and metastable state is less than half a water molecule. The present data indicate that the more hydrophylilc galactocerebrosides show a higher resistance for dehydration in the rigid state and a lower one for solvation by ethylene gycol than the glucocerebrosides. We can not determine 'unfreezable water' bound to the liquid-crystalline phase but the larger area per molecule should allow for more hydration. If dehydration is cooperative and large clusters are dehydrated to form nuclei for transfer to the rigid state, this process can be aided by lower water activity if it affects equally the two phases. The process may depend also on the affinity of the head groups to water and to ethylene glycol in the liquid-crystalline and in the rigid-crystalline states. In this light one has to see the difference between the behavior of the gluco- and of the galactocerebrosides. In pure water the hydration difference between the liquid crystalline and the rigid states is large in all the cerebrosides. At high concentration of ethylene glycol, the solvation difference of glucocerebrosides in the liquid-crystalline and in the rigid states is is relatively small allowing fairly rapid nucleation at moderate undercooling. The galactocerebrosides retain a larger hydration differences between the liquid-crystalline and the rigid state, which impedes nucleation and preserves undercooling. High degrees of undercooling are stable for long times before dehydrated nuclei are formed.

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